FREEZING RESISTANCE IN ANTARCTIC AND ARCTIC FISHES: ITS RELATION TO MODE OF LIFE, ECOLOGY AND EVOLUTION *

by

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ABSTRACT. - Biological antifreezes are present in most Antarctic and Arctic fishes and lower the freezing point of most of their body fluids below the freezing point of seawater (-1.9°C), without substantially increasing their osmotic pressure. In the blood of Antarctic notothenioid and Arctic gadiform fishes, freezing is inhibited by antifreeze glycopeptides (AFGP). These antifreeze molecules are built up of repeating tripeptide units (Ala-Ala-Thr)n, to which the disaccharide β-D-galactosyl-(1->3)α-N-acetyl-D-galactosamine is linked through the hydroxyl oxygen of the threonyl residue. Species of Liparididae, Zoarcidae, Cottidae and Pleuronectidae in the Antarctic, Arctic and northern-boreal waters synthesize only unglycosylated antifreeze peptides (AFP). The level of antifreeze concentration was dependent on the ambient water temperature, the depth of distribution, the life history and the level of activity of the species. Whereas the synthesis of AFGP in Antarctic and Arctic fish species appear to regulate by the ambient water temperature, the AFP production in Arctic fish and probably in non-notothenioid Antarctic fish will be controlled by photoperiod. Surprisingly, detectable concentrations of AFGPs in perciform fish of the Antarctic and gadiform fish of the Arctic and Antarctic could illustrate, that before the continental drift occurred a precursor glycopeptid existed, and that the existence of freezing resistance in some species reflects the past glaciation. The wide distribution and high heterogeneity of AFPs point to the assumption that these peptides are results of cold shock stress responses in a different way.

RÉSUMÉ. - Résistance à la congélation des poissons antarctiques et arctiques: son rôle en rapport avec le comportement, l'écologie et l'évolution.

Les antigels biologiques sont présents chez la plupart des poissons antarctiques et arctiques et abaissent le point de congélation de presque tous les fluides internes en-dessous du point de congélation de l'eau de mer (-1,9°C), sans pour autant augmenter la pression osmotique. Dans le sang des Notothenioidei antarctiques et des Gadiformes arctiques, la congélation est inhibée par des glycopeptides antigel (AFGP). Ces molécules antigel sont formées par des unités tripeptidiques répétitives (Ala-Ala-Thr)n, auxquelles le disaccharide β-D-galactosyl-(1->3)α-N-acetyl-D-galactosamine est relié grâce à un atome d'oxygène hydroxylé. Les espèces de Liparididae, Zoarcidae, Cottidae et Pleuronectidae des eaux antarctiques, arctiques et nord-boréales ne synthétisent que des peptides antigel non-glucosylés (AFP). Le niveau de concentration des antigels dépend de la température ambiante de l'eau, de la profondeur, de la biologie et du niveau d'activité des espèces. Tandis que la synthèse des AFGP chez les espèces antarctiques et arctiques semble régulée par la température de l'eau ambiante, la production d'AFP chez les poissons arctiques, et probablement chez les poissons antarctiques non Notothenioidei, est contrôlée par la photopériode. Curieusement, des concentrations détectables d'AFGP chez les poissons Perciformes antarctiques et chez les Gadiformes arctiques et antarctiques pourraient être le signe qu'un glycopeptide précurseur était présent avant que les continents ne se déplacent, et que l'existence d'une résistance à la congélation pourrait refléter les glaciations passées. La vaste réparti-

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tion et la grande hétérogénéité des AFP semblent être la preuve que la production de ces peptides est le résultat d'une réponse au stress dû au froid.

Key-words. - Notothenioidei, Gadiformes, PS, Antarctic Ocean, PN, Arctic Ocean, Antifreeze, AFGP, Evolution, Mode of life, Activity, Metabolism.

A variety of physical characteristics of cold sea-water has certainly influenced the evolutionary adaptation of the fish fauna in the Arctic and Antarctic Oceans. However, it must be considered that over 90% of the global sea-water is below 5°C. During the winter season the polar oceans and the near shore waters of the north temperature oceans are at the freezing point of seawater (-1.9°C), a temperature well below the freezing point of a typical marine teleost (-0.8°C) (Black, 1951). In the presence of ice, supercooling or undercooling is lethal, and therefore a one degree difference between the freezing point of the fishes body fluids and the environment will lead to freezing (Scholander *et al.*, 1957) because parts of the fishes integument and gills do not constitute a barrier to the propagation of ice. Even partial freezing of fishes has been shown in all cases to lead to death, although several hours may elapse between thawing and death (Scholander *et al.*, 1957). In the absence of ice, fishes can undercool by several degrees, and in the fjords of Labrador bottom fishes have been reported to be undercooled by 1°C and spend their lives in this metastable state (Scholander *et al.*, 1953; Fletcher *et al.*, in press). Therefore, for marine organisms cold water is not an unusual environment (Clarke, 1987) and low temperature as such is unlikely to be a limiting factor for biosynthetic processes.

Much of the polar oceans is perennially at its freezing point of -1.9°C and is ice-laden in its shallow waters (DeVries and Eastman, 1982). Many fishes live in this environment and frequently come into contact with ice (Eastman, 1993), yet do not appear to freeze. In fact some use the abundant ice crystal formations as a habitat and spend their entire lives there to forage for food and to escape predators but do not freeze under these conditions. Freezing in these fish species does not occur unless they are exposed to ice at temperatures below -2.2°C, in which case the result is death (DeVries and Lin, 1977).

In fishes inhabiting freezing environments, concentrations of sodium chloride in the body fluids are slightly higher than in temperature marine fishes and are responsible for 40-50% of the observed freezing point depression (Duman and DeVries, 1975). In Antarctic fishes, concentrations of other ions (Dobbs and DeVries, 1975) and small organic solutes (DeVries, 1968) are similar to those found in temperate water fishes. In these fishes the additional freezing point depression of the blood has been shown to be associated with solutes in the colloidal fraction of the blood, as indicated by the observation that over half of the blood freezing point depression is retained by a dialysis membrane with a molecular weight cut off of 3000 (DeVries, 1982; Woehrmann, 1995). The association of the colloidal fraction and the large freezing point depression indicates that relatively large solutes are involved and also implies that these solutes exert their effect by a non-colligative mechanism (DeVries, 1988).

It took almost 10 years before the nature of these freeze-protecting plasma solutes was discovered by DeVries and Wohlschlag (1969) and characterized by DeVries *et al.* (1970). We now know that they are polypeptides, or glycopeptides, which are primarily synthesized in the liver and secreted into the blood. On a mass basis both the glycopeptides and peptides are nearly as effective as sodium chloride in depressing the freezing point of water (DeVries, 1984). Still collectively termed "antifreeze", these (glyco)peptides

depress the freezing point by 200-300 times more than that expected on the basis of colligative relations alone (DeVries, 1971a, 1971b). Thus, fish have evolved a mechanism to reduce the freezing point of their body fluids without appreciably changing the osmotic properties of these fluids.

BIOLOGICAL ANTIFREEZES IN POLAR FISHES

Glycopeptide and peptide antifreeze compounds have evolved in a number of unrelated lineages of cold water teleosts including notothenioids, zoarcids, cottids, gadids, pleuronectids, clupeids and osmerids (Kao *et al.*, 1986; Davies *et al.*, 1988; DeVries, 1988; Ewart and Fletcher, 1990; Cheng and DeVries, 1991; Woehrmann, 1996).

Five distinct antifreeze peptide classes have been found to date (Table I). They are either glycopeptides or peptides. These blood serum antifreeze types consists of a set of closely related peptides resolvable by high performance liquid chromatography (HPLC) into as few as 2 and as many as 12 independently active components (DeVries, 1988; Davies and Hew, 1990; Woehrmann and Haselbeck, 1992; Woehrmann, 1995). The non-glycosylated antifreeze peptides are common in Antarctic and Arctic fishes. In table I, AFP I refers to the alanine rich (60 mol%) antifreeze peptides typified by those of the winter flounder (*Pleuronectes americanus*). This antifreeze has been crystallized and found to be completely α helical (Yang *et al.*, 1988; Sicheri and Yang, 1995). The antifreeze peptide type II from the sea raven (*Hemitripterus americanus*), smelt (*Osmerus mordax*) and Atlantic herring (*Clupea harengus harengus*) is characterized by disulphide bridges and an extensive β-structure. These AFP share structural homology with the carbohydrate recognition domain of C-type lectins (Ng *et al.*, 1986; Ewart and Fletcher, 1990; Ewart *et al.*, 1992). In addition, the AFP from the herring and smelt are dependent on Ca^{2+} for activity (Ewart *et al.*, 1996).

The third type of peptide antifreeze found to date (AFP III) is common in zoarcids of the Antarctic and Arctic (Hew *et al.*, 1984). In *Rigophila dearborni*, there are seven separate antifreeze peptides all of which appear to be identical in size, while in *Austrolycichthys brachycephalus* there are three peptides. The molecular weights of the *R.*

Table I. - Structural characteristics of antifreeze peptides and glycopeptides.

	PAGP	AFGP	AFP I	AFP II	AFP III
Carbohydrate	Yes	Yes	No	No	No
Mass (kDa)	120	2.6 to 33	3.3 to 4.5	11 to 24	6.5
Primary structure	hyaluronic acid glycine-rich	(Ala Ala Thr)n disaccharide	alanine-rich	cystine-rich	general
Secondary structure	α helical	expanded	α helical	β sheet	β sandwich
Tertiary structure	n.d.	n.d.	100% helix	n.d.	n.d.
Biosynthesis	n.d.	multiprotein	prepro AFP	prepro AFP	pro AFP
Protein components	2	8	7	2-6	12
Gene copies	n.d.	n.d.	80-100	15	30-150
Fish species	*Pleuragramma*	Notothenioidei Gadiformes	Pleuronectidae Cottidae	Cottidae Clupeidae	Zoarcidae

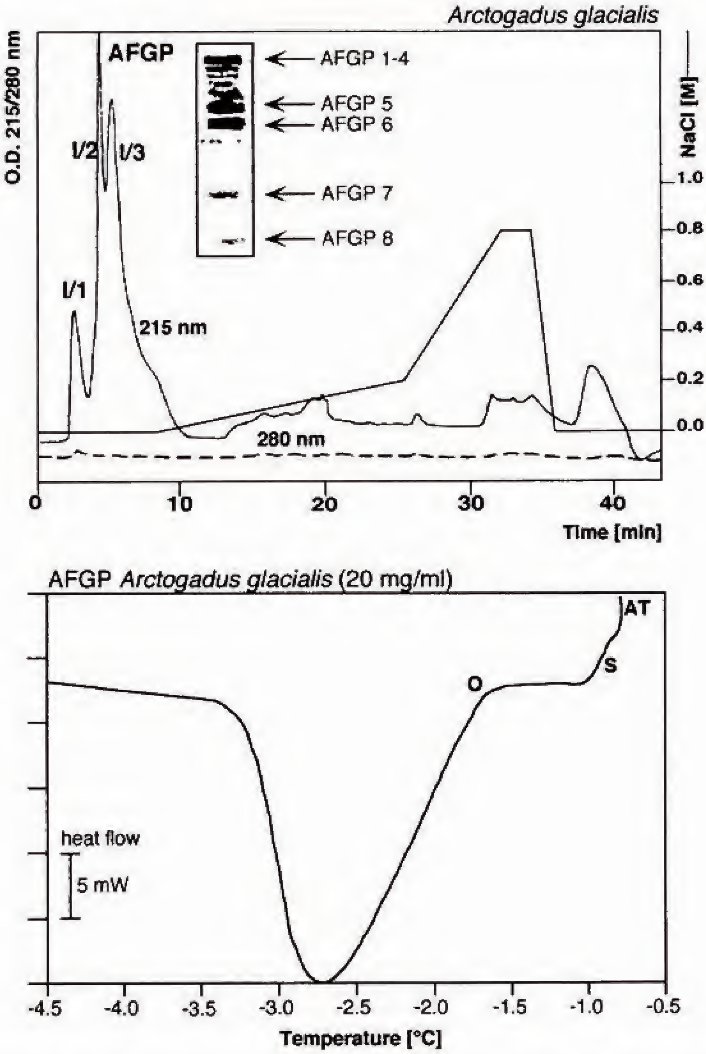


Fig. 1. - Elution profile on DEAE anion-exchange HPLC of antifreezes from the gadiform *Arctogadus glacialis*. One ml of protein-solution (1 mg.ml^{-1}) was injected. Starting buffer of 20 mM Tris-HCl, pH 9.5 with a salt-concentration gradient (0-0.8 M NaCl); absorbance at 215 and 280 nm. Pooled fractions (peak I/1-I/3) run on SDS-polyacrylamide gel electrophoresis. Antifreeze glycopeptides were blotted on nitrocellulose membrane and detected by the lectine peanut agglutinine (PNA) recognize the disaccharid moiety of AFGP (Woehrmann, 1993). The antifreeze glycopeptides consists of a series of eight distinct glycopeptides ranging in molecular weight from 33,700 to 2668 daltons (AFGP 1-8).

dearborni peptides as calculated from their primary structures are approximately 6900 dalton. The amino acid composition of the Antarctic eel pout is not unusual, consisting of a nearly even distribution of most amino acids except histidine, cystine, and two of the aromatic amino acids, phenylalanine and tryptophan (Schrag *et al.*, 1987). AFP III has no bias in its amino acid composition and contains a β-sandwich secondary structure (Sonnichsen *et al.*, 1993).

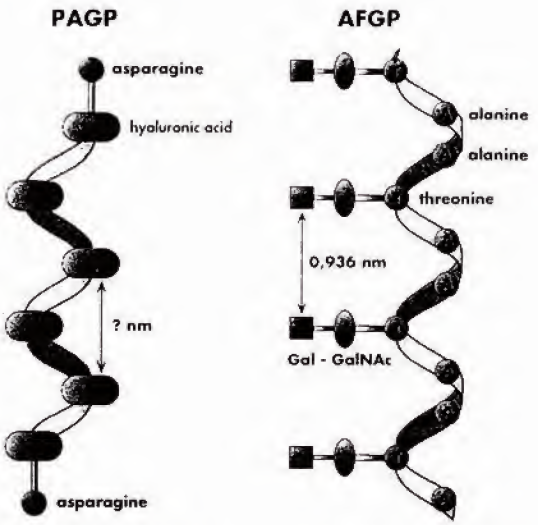


Fig. 2. - Model of the secondary structure of the antifreeze glycopeptides (AFGPs) of notothenioids and of the *Pleuragramma*-antifreeze glycopeptide (PAGP). The AFGP consists of amino acids in the sequence [alanyl-alanyl-threonine]$_n$. Each threonine is joined to a disaccharide through a glycosidic linkage. In low molecular weight AFGPs 6-8 (MW 7900 - 2668 dalton), proline is periodically substituted for alanine at position one of the tripeptide. The PAGP with the disaccharide unit hyaluronic acid consists of D-glucoronic acid and N-acetyl-D-glucosamine with a β(1-3) glycosidic linkage. The peptides are presumably linked to the glycan by asparagine through a N-glycosidic linkage.

The fourth type comprises the antifreeze glycopeptides (AFGP) found in the Antarctic notothenioids and in the Arctic cods - the Gadidae (Fig. 1). The AFGP consists of a series of eight distinct glycopeptides ranging in molecular weight from 2600 to 33,700 daltons. For convenience they are numbered and classified as large (1-5; 33.7 - 10.5 kDa) and small (6-8; 7.9 - 2.6 kDa) molecules. The primary structure of these glycopeptides of all investigated species is made up of a repeat of the tripeptide unit alanyl-alanyl-threonine, in which the disaccharide β-D-galactopyranosyl-(1-3)-2-acetamido-2-deoxy-α–D-galactopyranose is attached to the hydroxyl oxygen of the threonine residue through a O-glycosidic linkage (Komatsu *et al.*, 1970; Shier *et al.*, 1975; Feeney and Yeh, 1978) (Fig. 2). In smaller sized AFGPs proline replaces some of the alanine residues.

The fifth type of biological antifreeze was found only in *Pleuragramma antarcticum* (Woehrmann and Haselbeck, 1992; Woehrmann *et al.*, unpubl. data). The *Pleuragramma*-antifreeze glycopeptide (PAGP) was shown in the biochemical analysis to be a proteoglycan bearing carbohydrate chains of the hyaluronic acid type (Fig. 2). Hyaluronic acid usually consists of 250 to 25,000 kDa disaccharide units, held together by glycosidic β(1-4) links. The basic disaccharide unit consists of D-glucoronic acid and N-acetyl-D-glucosamine with a β(1-3) glycosidic linkage. Hyaluronic acid as a polyanion binds to cations. This explain the strong binding affinity of PAGP to the DEAE anion exchange chromatography (Woehrmann and Haselbeck, 1992; Woehrmann, 1995). X-ray structural analysis of fibres has shown that Ca^{2+} hyaluronate forms expanded left-handed single strain helices, containing three disaccharide units per turn. This secondary structure could explain the extraordinary freezing activity. On the other hand, similarities to AFGPs become obvious (left-handed helices composed of glycopeptides). The peptides, consis-

ting of 30.1 mol% glycine, 14.7 mol% aspartic acid, 15.5 mol% glutamic acid, 11.3 mol% alanine and presumably linked to the glycan by Asn-GlcNAc, confer a certain flexibility to the, otherwise rigid, hyaluronic acid molecule.

MECHANISM OF BIOLOGICAL ANTIFREEZE EFFECT

Unlike the low molecular weight cryoprotectants (e.g., glycerol) of cold hardy insects (Zachariassen, 1985), AFGPs lower the freezing point in a noncolligative manner. Since their effectiveness is not strictly dependent on the number of particles in solution, they do not upset osmotic gradients within the fish. The antifreeze activities of the AFGPs ranges from about 0.52°C-1.20°C at a concentration of 20 mg/ml; on a molar basis this represents about 100-300 times the (thermodynamic) freezing point depression expected on the basis of particle number. Thus high molecular weight AFGPs are most effective in lowering the freezing point, probably because their size ensures a more efficient adsorption to ice (Raymond and DeVries, 1977). The small molecular weight forms of AFGP 7-8 (MW 2668 and 3274) comprise most of the circulating antifreeze (Burcham *et al.*, 1984) but show only two-thirds of the antifreeze activity of the larger AFGPs (Ahlgren and DeVries, 1984). The chemical structure of these AFGPs is highly conserved among all high-Antarctic notothenioids studied to date (Ahlgren and DeVries, 1984; Woehrmann, 1996, 1997a).

Studies on the purified AFGPs revealed that chemical modifications (by acetylation, periodate oxidation and complexation with borate), or the removal (by alkaline β-elimination and by digestion with endo-α–N-acetylgalactosaminidase) of the sugar residues result in a loss of antifreeze activity. Similarly, inactivation of PAGP was observed following splitting of the carbohydrate chain hyaluronic acid and the peptide group by the enzyme hyaluronidase. Furthermore, the peptide is split off the glycan structure by means of hydrazinolysis (Woehrmann *et al.*, unpubl. data). For lowering the freezing point both the glycan and the peptide structure are necessary.

The various forms of antifreeze peptides and glycopeptides vary considerably from each other in numerous molecular and biochemical aspects other than size so that some of these differences probably contribute to broad range in antifreeze activity. All of the glycopeptide and peptide antifreezes exhibit the same depression of the freezing point of water on a weight basis, except for the PAGP and the smaller glycopeptides 7 and 8, which are found in the Antarctic nototheniids and Arctic gadiforms (Lin *et al.*, 1972; Woehrmann, 1993) (Fig. 3). The freezing point of a 4% antifreeze solution is -1.3°C while the melting point of the ice in it is approximately -0.02°C.

On a mass basis, AFGP from *Pleuragramma* AFGP exhibited the highest while AFGP from *Neopagetopsis* showed the lowest activity. The activity of the antifreeze glycopeptide of *Muraenolepis marmoratus* (probably AFGP) and of the proteoglycan PAGP (*Pleuragramma*-antifreeze glycoprotein) were considerably lower than all of the AFGP considered. The antifreeze activity of *M. marmoratus* is essentially the same as that reported for the low molecular weight AFGP isolated from notothenioids (Knight *et al.*, 1984; Woehrmann, 1993). The antifreeze activity curve observed for *Pleuragramma* AFGP is comparable to that reported for high molecular weight AFGPs of other notothenioids such as *Dissostichus mawsoni* (Schrag *et al.*, 1982; Knight *et al.*, 1984) and to the curve reported for the AFP of the Arctic ocean pout (Kao *et al.*, 1986).

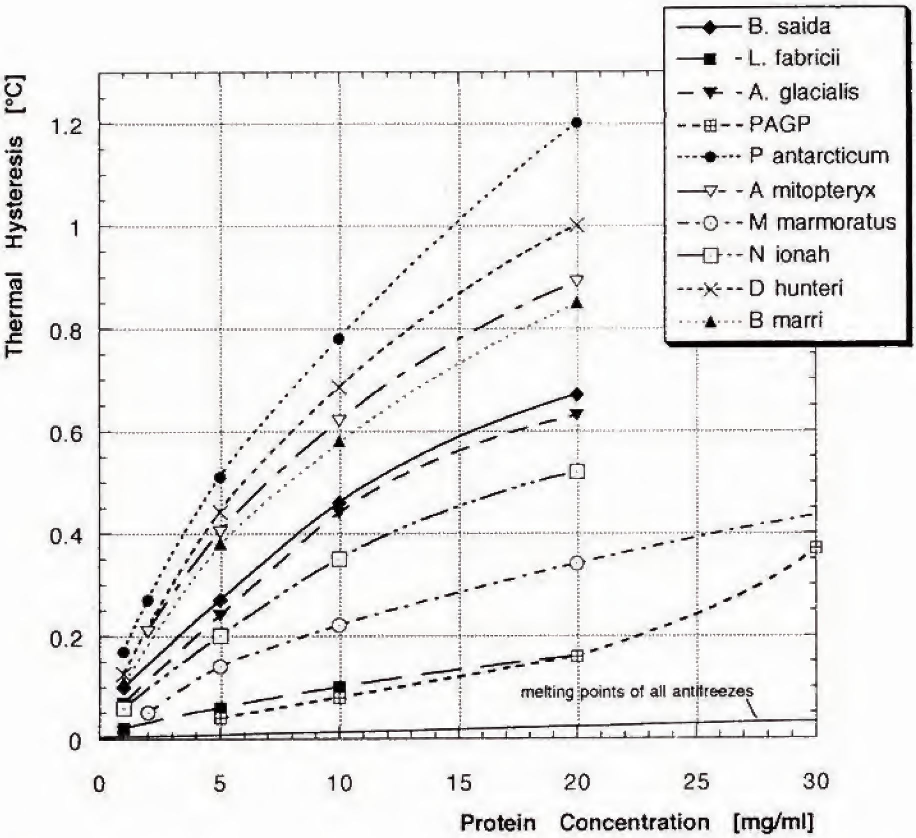


Fig. 3. - Comparison of thermal hysteresis curves on a weight basis of antifreeze peptides and glycopeptides obtained from *Boreogadus saida* (AFGP), *Liparis fabricii* (AFP, not further characterized), *Arctogadus glacialis* (AFGP), *Pleuragramma antarcticum* (PAGP and AFGP 1-5), *Aethotaxis mitopteryx* (AFGP), *Muraenolepis marmoratus* (probably AFGP), *Neopagetopsis ionah* (AFGP), *Dacodraco hunteri* (AFGP), and *Bathydraco marri* (AFGP). The melting points are the same for all the antifreeze solutions.

However, if the freezing points are compared on a molar basis then it appears that the depressions of the freezing point by the AFGPs of Antarctic and Arctic species are correlated with the size of the AF molecule, with the largest one showing the greatest effect (Schrag *et al.*, 1982; Woehrmann, in press) (Fig. 4). This same relationship has also been shown to exist with the AFPs in a few north-temperature fishes (Kao *et al.*, 1986). However, AF molecules smaller than a certain size lose the unique antifreeze effect, and their effect on freezing point becomes strictly colligative. For both peptides (AFP) and glycopeptides (AFGP and PAGP), this loss of antifreeze effect occurs when molecules are reduced in size below a molecular weight of approximately 1500 dalton (Schrag *et al.*, 1982; Woehrmann *et al.*, unpubl. data). Hew *et al.* (1985) have demonstrated that the two major peptides (MW 1400 and 2100) yielded by digestion of sculpin AFP were devoid of activity. The smallest active AFGP found to date is the AFGP 8 with a MW of 2668 Da, determined by plasma desorption-mass spectrometry (Woehrmann, 1995).

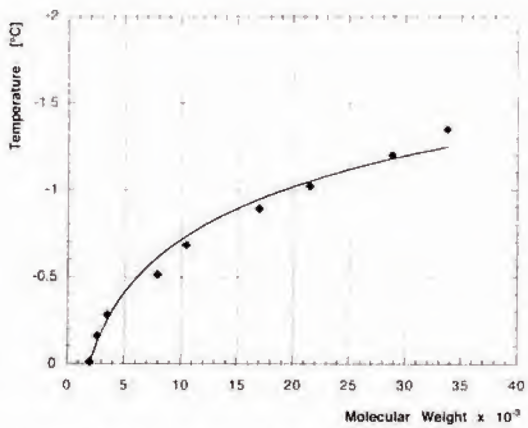


Fig. 4. - Relationship between freezing point and molecular weight of the antifreeze glycopeptides AFGP 1-8 isolated from Antarctic notothenioids and Arctic gadiforms. All concentrations are 1 mM. The data point at 2000 was obtained from antifreeze glycopeptide 8 which was shortened by one glycotripeptide unit by Edman degradation.

The AFGP from *Pleuragramma antarcticum*, *Dacodraco hunteri*, *Aethotaxis mitopteryx*, *Cryodraco antarcticus*, *Chaenodraco wilsoni*, and *Neopagetopsis ionah* are structurally homologous, having an amino acid composition of approximately 60% alanine, the disaccharid moiety Gal-GalNAc, and being largely helical structure. Therefore, it is likely that their differences in activity can be attributed to both chain length as well as molecular weight. Hew *et al.* (1985) observed that within AFP I (Fletcher *et al.*, in press) a decrease in chain length is associated with reduction in α–helical content and the number of specific amino acid repeats within their primary structures. This amino acid repeat structure is the major functional domain for antifreeze activity. Kao *et al.* (1986) concluded that within the different classes of AFP, activity increases with increasing molecular weight (3300 to 9700 dalton). The specific antifreeze activity per mg protein decreases when the peptides have MW of 4000 dalton and higher. Therefore, on a molecular weight basis, there appears to be no advantage in evolving large AFP molecules to achieve a freezing resistance required by the northern temperate fish species. In contrast, AFGPs and PAGP do not attain the antifreeze activity of the AFP until they reach a molecular weight greater than 8000 dalton. Judging from the level of freezing resistance required by the Antarctic notothenioids, the large AFGP and PAGP in the special case of *P. antarcticum* molecules are essential to their survival in ice-laden water.

All AFGP components examined have a plateau in activity at high concentration; the actual value of the plateau activity, however, differs between the different AFGP components depending on their length. While the low molecular weight components lose activity at deep supercooling, very high concentrations can restore the activity (Burcham *et al.*, 1986). The activity data fit a reversible kinetic model of AFGP activity, and the coefficients obtained can be used to compare the activity differences between antifreeze components (Feeney, 1988). The model was also shown to describe the activity of the thermal hysteresis protein of the insect, *Tenebrio molitor*, and was useful for studying the mechanism of the *P. antarcticum*-specific PAGP.

Circular dichroism (CD) studies have shown that at low temperatures AFGP does possess an ordered structure and this is converted to a random coil structure at high tempe-

ratures or in the presence of high concentrations of $CaCl_2$ which is known to destroy ordered structures in polypeptides such as polyproline and in proteins (Hennessey and Johnson, 1981). The contribution of the sugar moiety to the observed CD spectra can be negligible. Differences in the activities of low and high molecular weight AFGP are due to the difference in their sizes and not due to variations in their conformation (Rao and Bush, 1987).

Furthermore, the loss of activity of the low molecular weight AFGP 8 on its own could be due to its inability to adequately cover ice growth sites. It is possible that cooperativity between high and low molecular weight AFGP may occur directly on the ice surface. In contrast, no cooperativity was observed at temperatures of nucleation < -3°C between the high-alanine AFP of *Pseudopleuronectes americanus* and AFGP 8, even though additive effects were observed at temperatures of nucleation > -1°C (Feeney, 1988).

WATER DEPTH AND SYNTHESIS OF BIOLOGICAL ANTIFREEZES

In the high-Antarctic shallow water species are abundant only above 400-450 m depth. The benthic/epibenthic *Cygnodraco mawsoni*, *Trematomus pennellii* and *Pagetopsis macropterus* are representatives of this group, found to possess high amounts of both low and high molecular weight AFGP. Eurybathic species are abundant over most of the depth range down to 600-700 m. *Pogonophryne scotti*, *Racovitzia glacialis*, *Trematomus lepidorhinus* and *Cryodraco antarcticus* belong to this group. The antifreeze content in these species is moderate, in active species (*C. antarcticus*) lower than in sluggish species (*P. scotti*). Deeper shelf and upper slope species occur only at depths below 400-450 m. *Lepidonotothen kempi*, *Dacodraco hunteri* and *Muraenolepis marmoratus* in the Lazarev Sea, *Bathydraco marri* and *Trematomus loennbergii* in the Weddell Sea are examples of this group. The Lazarev Sea species have lower amounts of antifreezes which is likely related to the higher water temperature. On contrast, *D. hunteri*, caught in the Lazarev Sea, synthesize one of the most effective AFGP (1.0°C at 20 mg/ml) with relatively high amounts of high molecular weight AFGP due to their distribution in colder areas such as the southern Weddell Sea.

Little is known about the bathymetric range of the deep-sea bottom and benthopelagic fish fauna. Some notothenioids, such as *Pogonophryne macropogon* and *Bathydraco antarcticus*, as well as a number of zoarcids and liparidids have been found as deep as 2000-2600 m and could be referred to as deep-sea rather than deeper shelf and upper slope species. They do not require any antifreeze protection owing to the water depth and temperature of their habitat. Nevertheless, these benthic and sluggish species still possess antifreezes in although small amounts. Antifreezes are the non-glycosylated AFP in the case of zoarcids and liparidids, and the AFGPs in *Pogonophryne* spp. and *Bathydraco* spp. Moreover, *Bathydraco* spp. distributed in the cold water of the Filchner Depression, synthesize additional antifreeze peptides in the same concentration as the AFGP. Antifreeze activity is 0.84°C at a peptide concentration of 40 mg/ml. So far, these peptides could not be further characterised (Woehrmann, 1993).

The measurable thermal hysteresis (0.1°-0.5°C) of blood serum of several deep-living species (e.g., *Muraenolepis marmoratus*, *Paraliparis somovi*, *Neopagetopsis ionah*, *Bathydraco* spp.) suggests that the serum contains modest amounts of noncolligative antifreezes, but that this does not lower the freezing point of serum sufficiently to

protect these species from freezing at the usual -1.9°C surface temperature in the Shelf Surface Water (SSW). Combined with elevated osmolality of body fluids, a widespread phenomenon in polar teleosts (O'Grady and DeVries, 1982), the freezing point depression of serum in deep water species is only 0.98°-1.38°C below the freezing point of pure water.

However, the freezing behaviour of the serum of *Paraliparis somovi* is similar to that reported for some northern boreal species (Raymond, 1989; Woehrmann, 1993). The freezing points are not well defined and the ice growth in the serum progresses through periods of stops and starts as the temperature is lowered. In other non-notothenioid species from the Lazarev Sea (e.g., *Macrourus holotrachys* and *Lycenchelys hureaui*) freezing point depression of serum could not be detected due to the extremely low concentrations of the antifreeze peptides (Woehrmann, 1993).

While antifreeze protection down to -2.2°C is necessary for fishes inhabiting relatively shallow ice-laden water, most non-notothenioid species and some notothenioids (e.g., *Neopagetopsis ionah*) live at 600 m depth and deeper, a habitat with different physical parameters and less ice (with the exception of the southern Weddell Sea and Filchner Depression). Seawater at the surface of the Weddell Sea freezes at approx. -1.86°C. Because of the pressure effect on the freezing point (0.00753°C/10 m; Lewis and Perkin, 1985), seawater at 500 m depth with a salinity of 34.7‰ freezes *in situ* at -2.24°C. Hence, based on the freezing point of serum, the freezing point would be -1.50°C in *Paraliparis somovi* (623 m), -2.28°C in *P. scotti* (830 m), -1.90°C in *N. ionah* (799 m), and -2.36°C in *Bathydraco antarcticus* (140 m).

Fishes living at these depths and temperatures in the eastern Weddell Sea and the Lazarev Sea are in little danger of freezing because there is no ice in the water. Some of these species were thought to lack antifreeze, remain in a supercooled state in this deep water habitat instead (DeVries and Lin, 1977). In the presence of ice, however, supercooling is not possible as the fishes gills and integument constitute no barrier to the propagation of ice in the body and even partial freezing leads to death (DeVries, 1988). In the southern Weddell Sea near the Filchner Ice Shelf the water at the undersurface of ice shelves is at its equilibrium freezing point. As this water flows out from under the shelf and rises, it becomes supercooled and potentially ice formation could occur at considerable depths. Large masses of single-crystal ice platelets were discovered in this area (Dieckmann *et al.*, 1986).

Beside the antifreezes in blood serum some Antarctic species have trunk skin as barrier to the entry of ice crystals into the body. Skin mucus of *Pogonophryne* spp. and *Pleuragramma antarcticum* contains amounts of AFGP, and in the case of *P. antarcticum* a mixture of different antifreeze types protect against freezing (Woehrmann, unpubl.). Asakawa *et al.* (1989) have isolated glycoproteins similar to AFGPs from the skin mucus of *Trematomus bernacchii*. A high water content of the subdermal extracellular matrix of *Paraliparis devriesi* does not require additional protection against freezing (Jung *et al.*, 1995). *In vitro* experiments on the skin of winter flounder (*Pseudopleuronectes americanus*) from Newfoundland support the hypothesis that the presence of epithelia exclude the entry of ice (Valerio *et al.*, 1992). The antifreeze concentration in the blood of some of these species is too low to protect against freezing. It is likely that the modified skin surfaces act as a physical barrier (Fletcher *et al.*, in press) to the propagation of ice into body fluids of sluggish species such as *Pogonophryne* spp., liparidids and probably in zoarcids and *Pleuragramma antarcticum*.

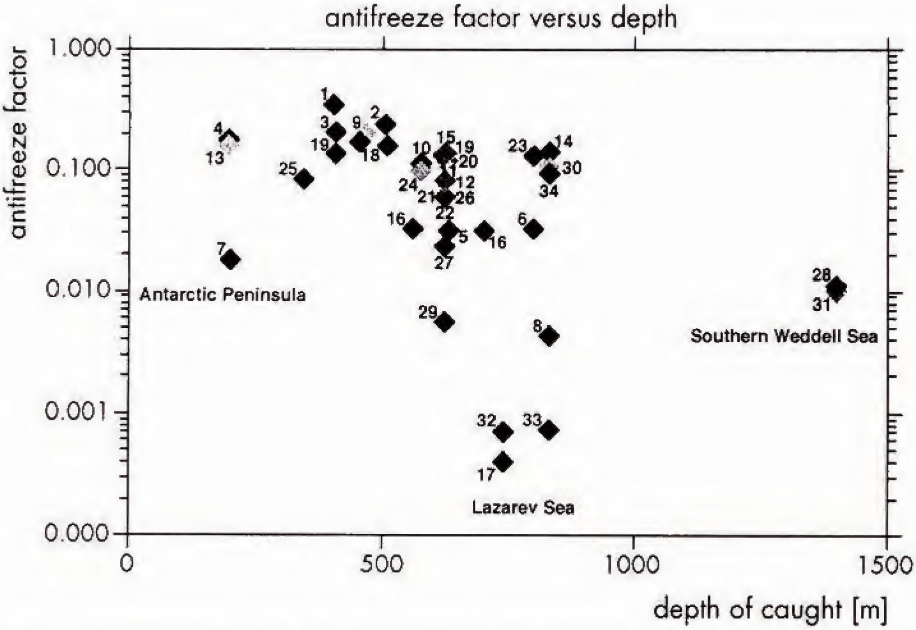


Fig. 5. - Antifreeze factor (total body concentration of antifreeze compounds x thermal hysteresis of purified antifreezes standardized to 20 mg/ml) versus the depth of caught. The shadow indicates the ambient water temperature, dark = cold water, light = warm water.
1 *Trematomus pennellii*, 2 *Pagetopsis macropterus*, 3 *Chionodraco hamatus*, 4 *Gymnodraco acuticeps*, 5 *Pleuragramma antarcticum*, 6 *Neopagetopsis ionah*, 7 *Gobionotothen gibberifrons*, 8 *Muraenolepis marmoratus*, 9 *Trematomus eulepidotus*, 10 *T. loennbergii*, 11 *Dissostichus mawsoni*, 12 *Trematomus bernacchii*, 13 *Cygnodraco mawsoni*, 14 *Pogonophryne scotti*, 15 *P. marmorata*, 16 *Aethotaxis mitopteryx*, 17 *Macrourus holotrachys*, 18 *Chaenodraco wilsoni* & *Pagetopsis maculatus*, 19 *Gerlachea australis* & *Chionodraco myersi*, 20 *Trematomus lepidorhinus*, 21 *Dolloidraco longedorsalis*, 22 *Cryodraco antarcticus*, 23 *Pogonophryne barsukovi*, 24 *Racovitzia glacialis*, 25 *Artedidraco loennbergi*, 26 *Dacodraco hunteri*, 27 *Bathydraco marri*, 28 *B. antarcticus*, 29 *Paraliparis somovi*, 30 *Pogonophryne permitini*, 31 *Bathydraco macrolepis*, 32 *Gymnoscopelus opisthopterus*, 33 *Lycenchelys hureaui*, 34 *Pogonophryne macropogon*.

In summary, antifreeze compounds are distributed in the blood serum and other body fluids. In some species they could be found in the skin mucus or in the subdermal extracellular matrix. In deeper living species the concentrations are too low to detect a thermal hysteresis in the blood serum. Furthermore, the thermal hysteresis depends on the relative composition of both high and low molecular weight AFGP. To take these facts into account and to compare the results of recent investigations of other authors with own results, the values about freezing resistance has been standardised. I have defined the **'antifreeze factor'** (AF) as the content of HPLC-purified antifreeze peptides and glycopeptides in % of the gutted fish × the maximum hysteretic effect (°C; standardised to 20 mg/ml) of these antifreezes.

The antifreeze factor versus the depth of minimum occurrence is shown in figure 5. As expected, the shallow water Antarctic (epi)benthic species such as *Trematomus pennellii*, *Pagetopsis macropterus* and *Gymnodraco acuticeps*, with a moderate activity have high concentrations of AFGP with a high hysteretic effect due to the high molecular

weight glycopeptides. Cryopelagic and active species such as the Antarctic Nototheniid *Pagothenia borchgrevinki* and the Arctic *Boreagodus saida* and *Arctogadus glacialis* also have high concentrations of AFGP in the blood. They are in danger to contact with ice crystals. These phenomena are resulting in highest AF values. Species distributed in warmer waters of the Lazarev Sea (e.g., *Muraenolepis marmoratus, Lycenchelys hureaui*) possess lower amounts of antifreeze with less thermal hysteretic effect compared with species (e.g., *Pogonophryne* spp.) of the same depth (830 m) in the Weddell Sea, resulting in a much lower AF value. Non-notothenioid species of the Lazarev Sea do possess some antifreezes in the blood serum and probably in the skin mucus, even though in extremely low concentration. A thermal hysteretic effect is not measurable with usually methods. With the antifreeze factor it is possible to compare this low values with antifreeze concentrations in other species. On the other hand, *Bathydraco* species have unusual high AF values. These species living in the extreme cold waters of the southern Weddell Sea need high concentrations of highly functionally antifreeze compounds. The antifreeze factor is a possibility to declare the antifreeze avoidance of Antarctic and Arctic fishes.

METABOLISM AND ANTIFREEZE PRODUCTION

There has been an evolutionary adaptation to overcome the rate-depressing effect of low temperature on the synthesis of proteins in notothenioids (Eastman, 1993). This is a important phenomenon as protein synthesis may constitute a significant portion of the maintenance metabolism and some enzymes, such as those involved in energy metabolism or synthesis of antifreeze peptides, require continuous replacement (Smith and Haschemeyer, 1980). For example, antifreeze glycopeptides account for 4% of the liver polypeptide synthesis and their half-life is approximately 10 weeks.

In fact, synthesising larger and apparently less efficient (lower activity per unit weight) molecules as in the case of the AFPs may be disadvantageous in terms of the metabolic cost involved. Although the metabolic costs required to synthesize and maintain active mature fish AFP and AFGP are unknown, some insight into relative energy costs can be gained by comparing the amounts of protein and peptide bonds (25 kcal/mol; Schulz and Schirmer, 1979) and peptide and carbohydrate bonds (4.4 - 5.8 kcal/mol; Toone, 1994) which are necessary to give the fish specific levels of freezing protection.

Zoarcids and some notothenioids (*Pogonophryne* spp. and *Bathydraco* spp.) are extremely sluggish (Woehrmann, unpubl. obs.) and have very low metabolic rates. Active notothenioid species such as *Trematomus bernacchii* show higher resting metabolism (Hubold, 1991). Scope for activity was therefore suggested to be one factor useful for determining the resting metabolic rates of Antarctic notothenioid fish (Wells, 1987).

The elevated resting metabolism of the notothenioid species may partly be due to the maintenance of an efficient nervous and sensory system and to the synthesis of sufficient levels of antifreeze peptides. Non-notothenioid species living in warmer and deeper water such as zoarcids, liparidids, and macrourids do not possess the high molecular and glycosylated antifreeze peptides of notothenioids and gadiforms and are not endemic Antarctic fish families. The investigation of specialised, endemic, yet sluggish notothenioid species can yield basic estimates on the metabolic cost for synthesizing antifreezes which are not directly related to activity.

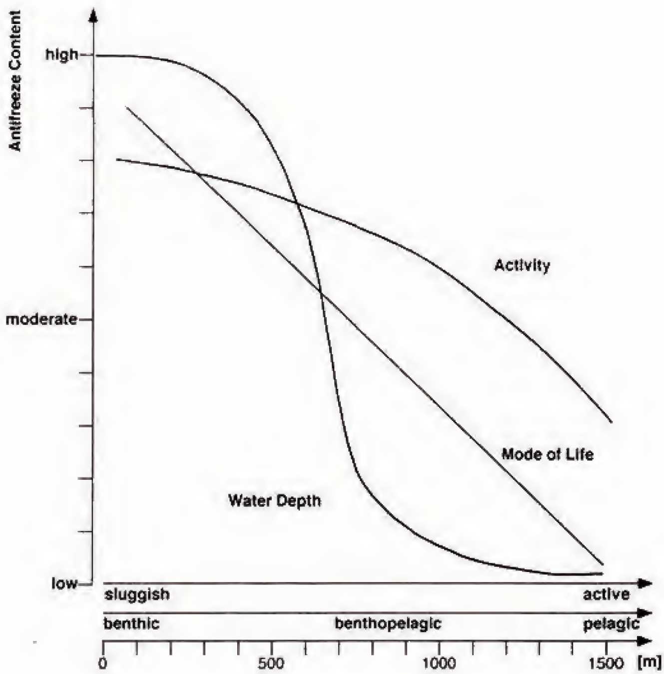


Fig. 6. - Antifreeze content in blood or other total body of investigated Antarctic and Arctic fishes in relation to their activity, mode of life and water depth.

Truly sluggish notothenioid species are *Pogonophryne* spp. and *Bathydraco* spp. All investigated species of both genera so far synthesize AFGP in relative high amounts (Woehrmann, in press). *Pogonophryne* spp. caught at 830 m depth and an ambient water temperature of + 0.75°C survived cooling to freezing point in the presence of ice crystals as observed in captivity (Woehrmann, 1990). Resting metabolism of *P. scotti* falls between that of zoarcids and nototheniids (Saint Paul *et al.*, 1988). In addition to the minimum metabolic rate of 10-20 mg $O_2kg^{-1}h^{-1}$ displayed by the zoarcid *Rhigophila dearborni*, *Pogonophryne scotti* respires approx. 15 mg $O_2kg^{-1}h^{-1}$ for metabolic purposes, which are not related to potentially high activity. A compilation of respiration rates of high Antarctic and Arctic fish shows a correlation with the activity pattern (Hubold, 1991) and the concentration of antifreezes in blood or other body compartments (Fig. 6). The difference in resting oxygen consumption between a sluggish zoarcid and a sluggish notothenioid may be a rough measure for the cost of maintaining high levels of glycosylated antifreeze peptides. However, more active species such as *Neopagetopsis ionah* living in the same environment (water depth of 800 m, water temperature above zero) possess lower amounts of AFGP.

It should be noted that AFGP synthesis is not continuous throughout the year in all Antarctic fish species as has been reported in earlier studies (O'Grady *et al.*, 1982). The content of antifreezes in *Pleuragramma antarcticum* depends on the fishes ontogenetic migration and hence is related to the ambient water temperature (Woehrmann, 1996). Early post-larvae and maturing adults, abundant near the ice shelf in the south-eastern

Weddell Sea, possess higher amounts of antifreezes than juvenile fish feeding on krill in the East Wind Drift.

Pleuragramma antarcticum occurs in epi- and mesopelagic layers and has a very high mean lipid content, second to *A. mitopteryx*. The lipids are deposited predominantly as triacylglycerols, and the lipid stores greatly improve the buoyancy characteristics of *P. antarcticum* (Eastman, 1993). Morphological, ultrastructural and blood physiological studies suggest, that pelagic Antarctic fishes like *P. antarcticum* and *A. mitopteryx* are rather sluggish with a low scope of activity and low metabolic requirements (Kunzmann, 1991; Woehrmann *et al.*, in press). In addition, the significantly increased viscosity of sea water at near-freezing temperatures and hence higher energy cost for swimming (Hubold, 1992; Eastman, 1993). Reduced energy requirements due to low metabolic rates have also been indicated by starvation experiments with the epibenthic *Trematomus eulepidotus*, which can survive extended periods without food (Woehrmann, 1988).

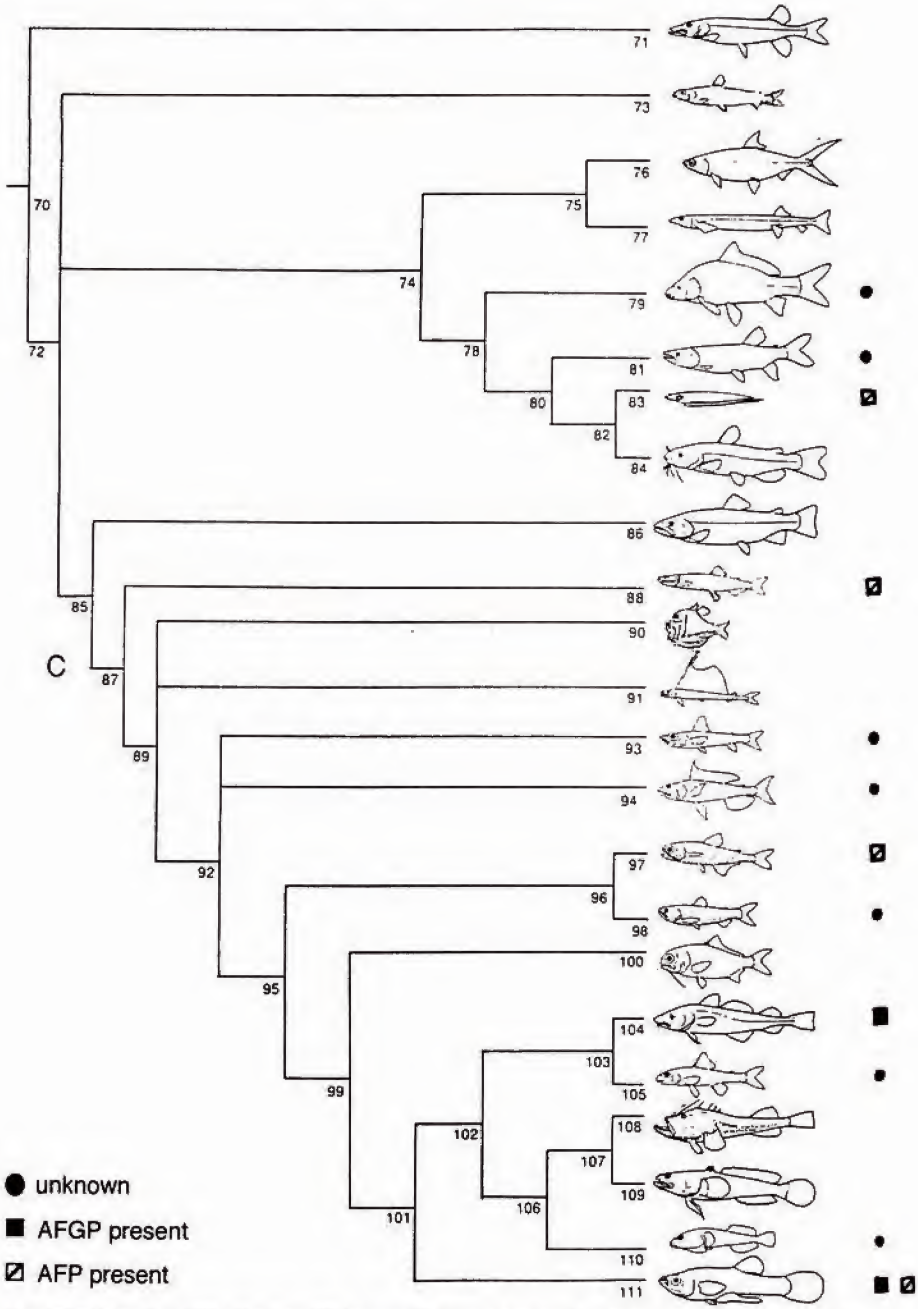
In conclusion, to minimise the metabolic cost for synthesizing antifreezes, the more active and/or pelagic species possess antifreeze peptides in a minimum essential concentration. However, extremely sluggish species with a low resting metabolism can synthesize antifreezes in higher amounts than they would need in their natural habitat due to the "sit and wait" foraging strategy and due to the year around food supply. Furthermore, the synthesis of different antifreeze glycopeptides and peptides in relative low concentrations and the highly functional mechanism of these compounds is an extraordinary adaptation which has developed *P. antarcticum* to minimise the energetic cost for a fully pelagic life in high-Antarctic waters.

THE EVOLUTION OF FISH AND BIOLOGICAL ANTIFREEZES

It is believed that notothenioids have evolved from a bottom living ancestral group after the establishment of the Antarctic Circumpolar Current (Eastman and Grande, 1989). Monophyly was assumed (Eakin, 1981; Iwami, 1985). However, antifreeze compounds were not essential for survival of the notothenioid stock and other fish groups during the rapid cooling at 38 million years ago or prior to the formation of the Southern Ocean at 25-22 million years ago. Most of the Southern Ocean was not cold enough to freeze fish (Eastman, 1993).

Antifreezes have been identified in all investigated high-Antarctic, high-Arctic and some northern boreal species (Eastman, 1993; Woehrmann, 1993, 1997b; Jung *et al.*, 1995; Fletcher *et al.*, in press). Our own studies have focused on the nototheniids, bathydraconids and channichthyids of the Weddell Sea and McMurdo Sound and on the pleuronectids and zoarcids of the Labrador Sea, with little attention devoted to other fish families (Fig. 7). Hence, antifreezes are probably a universal characteristic of notothenioids and gadiforms (and other fish groups), synthesized in significant concentrations only by those species which inhabit waters with subzero temperatures where ice is present or by species liable to encounter ice during latitudinal or vertical migrations.

The urinary loss of AFGPs in notothenioids (Eastman, 1993) and in the Arctic gadiform species *Boreogadus saida* (Christiansen *et al.*, 1996) is effectively circumvented by the evolutionary alteration of renal morphology. Species with significant amounts of AFGPs have aglomerular nephrons. The evolution of this aglomerular nephron in a small group of teleost fishes is the most significant departure from the basic renal pattern in vertebrates. In aglomerular nephrons, urine is produced by tubular secretion rather than by



e.g.: 70: Euteleostei; 82: Siluriformes; 88: Osmeroidei; 92 Eurypterygii; 96: Myctophiformes; 97: Myctophidae; 102: Paracanthopterygii; 104: Gadiformes; 111: Acanthopterygii (Scorpaeniformes, Perciformes, Notothenioidei)

Fig. 7. - Tracing of the evolution of different antifreeze glycopeptides and peptides. Phylogenetic topology is after Lecointre (1994). Circles indicate genera for which the presence of antifreeze peptides has not been tested.

filtration and small molecules such as AFGPs are spared from the filtration process (Eastman, 1993).

Eastman (1993) mapped the distribution of AFGPs and glomeruli on an area cladogram for the Notothenioidei. Together with the results of the present study it is evident that all species which possess AFGPs exhibit the apomorphic state of kidney. The plesiomorphic condition of kidney is retained by the non-Antarctic bovichtids without AFGPs. Species of the Nototheniidae distributed in extremely cold water (e.g., *Pagothenia borchgrevinki*) and in warmer water of the subantarctic area (e.g., *Dissostichus eleginoides*) have mixed character states for both AFGPs and glomeruli. The high-Antarctic subfamilies Trematominae and Pleuragramminae possess AFGPs at all and have aglomerular kidneys. On the other hand, the sub-Antarctic Eleginopinae and Nototheniinae are mixed in regard to these traits (Eastman, 1993).

I suspect that the distribution of notothenioids to the south and of the polar cod (*Boreogadus saida*) and the arctic cod (*Arctogadus glacialis*) to the north has been toward the acquisition of only the aglomerularism, and this trend can be correlated with phylogenetic diversification of notothenioids and gadiforms, and with inhabitation of colder water. The adaptation to aglomerularism so as to spare small molecules such as AFGPs from filtration is a necessary condition for species living in the high-polar waters to conserve the energy costs. Hence, it could be emphasized, that aglomerularism in such fish species is a key evolutionary innovation to survive in extremely cold waters. However, the ability to synthesize antifreeze peptides is not correlated to water temperature and therefore the synthesis of AFGPs is not a key innovation.

GENERAL CONCLUSIONS

The concentration and composition of antifreeze compounds appear to be dependent on the ambient water temperature, the depth of occurrence, the level of activity (resting metabolism), and the mode of life. Sluggish and benthic species, such as *Pogonophryne* spp. and *Bathydraco* spp., possess higher amounts of antifreezes as they would need in their natural habitat. Antifreeze concentration of more active as well as benthopelagic or pelagic species, such as channichthyids, *Pleuragramma antarcticum* and *Aethotaxis mitopteryx*, is exactly tailored to the ambient water temperature and depth due to the metabolic cost for synthesizing these glycopeptides and peptides. Freezing resistance is an important adaptation which all Antarctic fish species have successfully developed. Hence, the ability of species living in warm deep water to synthesize antifreeze indicate that antifreezes are ubiquitous. Furthermore low water temperature as such has not caused the paucity of non-notothenioid species in the Antarctic fish fauna. Rather, the limited shallow water habitat on the continental shelf, the glacial-interglacial periods and seasonal oscillation in the food supply are plausible ecological factors. Finally, the present time which represents a point of interglacial warm period in the evolutionary history of Antarctic fish species reflects that some species do not need a fully effective level of antifreeze but synthesize only minimum essential amounts.

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